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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Habener et al.	Examiner:	Wehbe, A.
Serial No.:	09/731,261		
Filed:	December 6, 2000	Group Art Unit:	1632
Titled:	Stem Cells of the Islets of Langerhans and Their Use In Treating Diabetes Mellitus	Conf. No.:	9060

DECLARATION UNDER 37 CFR 1.132 BY JOEL F. HABENER, M.D.

I declare:

1. I, Joel F. Habener hold an M.D. degree from the University of California, Los Angeles. I received my M.D. degree in 1965. My current positions are Investigator, Howard Hughes Medical Institute at the Massachusetts General Hospital, Associate Physician at the Massachusetts General Hospital and Professor of Medicine at Harvard Medical School. I have held the position of Howard Hughes Investigator since 1976. I have held the position of Associate Physician since 1989. I have held the position of Professor of Medicine at Harvard Medical School since 1989. Previously, I held the position of Associate Professor of Medicine at Harvard Medical School from 1975-1988. I am an inventor of the above-referenced patent application.

2. I have read the Office Action dated October 7, 2003, filed in the above-referenced patent application and understand that the Examiner has rejected claims 19-21 for allegedly being incomplete for lack of an essential step. The Examiner has stated that "the methods as claimed lack any step by which nestin-positive cells can be differentiated from the other cell types in the culture. Looking to the specification, the actual methods used to isolate nestin-positive cells include the essential step of removing floating cells from cells adhered to concanavalin A

coated plates."

3. My laboratory has developed a method for isolating a stem cell from a pancreatic islet of Langerhans that does not require a step wherein cells are cultured on concanavalin A coated plates.

4. Isolated human pancreatic islets were obtained through the JDRF Human Islet Distribution Program from the following centers: The Joslin Diabetes Center, Boston, the Northwest Tissue Center Islet and Cell Processing Laboratory, Seattle, and the Islet Distribution Center at the Diabetes Research Institute, Miami. Culture conditions were as follows. Single cell suspensions of Human pancreatic islet preparations were made by digestion with 5mg/ml Trypsin in PBS at 37°C and passage through a glass pipette. Viable cells were counted by Trypan Blue exclusion and seeded at 10,000 cells/cm^2 on tissue culture treated plastic dishes (Corning, Corning, NY). In initial experiments cell expansion was done in RPMI 1640 (11 mmol/l Glucose) (Invitrogen, Carlsbad, CA) with 10 mmol/l Hepes buffer, 1 mmol/l sodium pyruvate, 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 25ng/ml EGF, 20ng/ml bFGF and 1x penicillin/streptomycin. In later experiments CMRL 1066 medium (5.5 mmol/l Glucose) with 10% FBS, 1x penicillin/streptomycin, 100ng/ml beta nerve growth factor (β-NGF; R&D Systems, Minneapolis, MN), and 25ng/ml EGF was used. 24 to 48 hours after seeding, dead cells were removed by a media change and one wash with PBS. Thereafter, cells were expanded for 10-14 days until they reached confluence and medium was changed every 3 days.

According to this protocol, upon culture initiation only a few cells (less than 10%) attached to the dish and began to proliferate whereas the majority of cells did not attach to the dish and were washed off and discarded with the subsequent 2-3 changes of the culture media. Over 10-14 days of culture, the cells reached confluency. At this time, the levels of insulin mRNA in the cultured cells had markedly decreased compared to that of initial islets.

Immunostaining revealed only an occasional insulin-positive cell within the monolayer of expanded cells.

The resulting expansion cultures of progenitor cells contain at least two phenotypically distinct cell types, those that express nestin and vimentin and those that express epithelial markers cytokeratin 19 and E-cadherin, as detected by immunofluorescent staining as described below.

Expansion phase cells were grown on tissue culture treated plastic slides (Nalge Nunc, Naperville, IL). For immunostaining cells were fixed with PBS/4% paraformaldehyde for 10 minutes at room temperature (RT). Slides were blocked with normal donkey serum in PBS/0.1%Triton for 1 hour at RT, incubated with primary antibodies over night at 4°C, washed, incubated with Cy2/Cy3 labeled secondary antibodies for 1 hour at RT, washed again and mounted. In vitro generated cell clusters were embedded in a fibrin clot prior to fixation. Clotting was achieved by mixing solutions of human fibrinogen (80 mg/ml in PBS, Sigma, St. Louis, MO) and human thrombin (50 units/ml in 40 mmol/l $CaCl_2$, Sigma). Clots were fixed in 10% buffered formalin, dehydrated, embedded in paraffin and cut into 4μm sections. Sections were dewaxed in xylene, hydrated, boiled for 10 minutes in a microwave oven in 10mmol/l sodium citrate, pH 6 for antigen retrieval and stained as described above. Nuclei were counterstained with DAPI. Antibodies used were guinea pig anti-insulin (1:2,000), guinea pig anti-glucagon (1:2,000), rabbit anti-somatostatin (1:2,000), and guinea pig anti-pancreatic polypeptide (1:2,000; all from Linco, St. Charles, MS), rabbit anti human nestin (1:200, Chemicon, Temecula, CA), mouse anti vimentin (1:100, Signet, Dedham, MA) mouse anti smooth muscle actin (1:100, Sigma), mouse anti cytokeratin 19 (1:100, Sigma), mouse anti keratin (1:100, Chemicon), mouse anti desmin (1:100, Sigma).

Immunostaining revealed that expansion cultures consisted of two major types of cells: E-cadherin/cytokeratin 19 (CK19) positive epithelial cells growing in patches and vimentin/nestin-positive spindle-shaped cells growing separately from each other. Many of the

spindle-shaped cells also co-expressed smooth muscle actin. Occasional cells with epithelial characteristics (E-cadherin and CK19-positive) also stained positive for nestin (See Figure 1A-F, attached).

The two major populations of cells are easily separated based on differences in their morphologies. The nestin/vimentin positive spindle shaped fibroblatoid cells are markedly different from that of the E-cadherin/CK19 positive flat, cuboidal epithelial-like cells that are in patches. Under regular or phase contrast light microscopy, using low power, nestin/vimentin positive cells that are clearly separated from the E-cadherin/CK19 cells which are in distinct patches are selected. In certain embodiments, the nestin/vimentin positive cells are "cloned" by replating them and expanding them, multiple times.

The majority of the spindle shaped cells are nestin and vimentin positive as demonstrated by repeated immunohistochemical staining of expansion phase cultures, see Exhibit A.

Expansion phase cells were grown on tissue culture treated plastic slides (Nalge Nunc, Naperville, IL). For immunostaining cells were fixed with PBS/4% paraformaldehyde for 10 minutes at room temperature (RT). Slides were blocked with normal donkey serum in PBS/0.1%Triton for 1 hour at RT, incubated with primary antibodies over night at 4°C, washed, incubated with Cy2/Cy3 labeled secondary antibodies for 1 hour at RT, washed again and mounted. In vitro generated cell clusters were embedded in a fibrin clot prior to fixation. Clotting was achieved by mixing solutions of human fibrinogen (80 mg/ml in PBS, Sigma, St. Louis, MO) and human thrombin (50 units/ml in 40 mmol/l $CaCl_2$, Sigma). Clots were fixed in 10% buffered formalin, dehydrated, embedded in paraffin and cut into 4μm sections. Sections were dewaxed in xylene, hydrated, boiled for 10 minutes in a microwave oven in 10mmol/l sodium citrate, pH 6 for antigen retrieval and stained as described above. Nuclei were counterstained with DAPI. Antibodies used were guinea pig anti-insulin (1:2,000), guinea pig anti-glucagon (1:2,000), rabbit anti-somatostatin (1:2,000), and guinea pig anti-pancreatic polypeptide (1:2,000; all from Linco, St. Charles, MS), rabbit anti human nestin

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In view of the data presented above, one of skill in the art would accept that isolation of a stem cell from a pancreatic islet of Langerhans does not require a step wherein cells are cultured on concanavalin A coated plates.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

April 2, 2004
Date

Joel F. Habener
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predicts the ethological relevance of Mauthner-associated neural networks[31]. The 'commissural inhibitory network' normally suppresses both M-cells at low acoustic intensities and prevents the simultaneous firing of both M-cells[22]. Rapid pressure change provides a stimulus strength which saturates these inhibitory inputs and depolarizes both M-cells towards threshold. By ensuring greater inhibition of the M-cell contralateral to the sound source, the directional nature of particle motion could determine which cell fires first, and ensures the activation of one M-cell before the other. This activation inhibits the contralateral M-cell[23] and thus determines the direction of the escape movement[24,25]. This effectively preserves any directional information received from particle motion and functionally imposes it on the nondirectional pressure stimulus that initiates the escape. Thus, by discriminating between two forms of acoustic information, fish use separate components of the same stimulus to determine the onset time and direction of the behavioural response.

The presence of Weberian ossicles, a specialized structure connecting the swimbladder to the inner ear, seems to broaden the spectral range for hearing[12] in otophysan fishes[26] such as goldfish, and may also enhance the acoustic range of sound-initiated escape. Our experimental approach can be used both to study the neuroethological role of such specializations and to make comparative assessments of the relative hearing capabilities of various groups of fishes.

The M-cells are present in primitive fishes and pre-date the evolution of the swimbladder[27]. Thus, the successful expression of Mauthner-mediated escape could have influenced the evolution of the swimbladder for acoustic pressure transduction. Originally adaptive as a respiratory organ in hypoxic environments[28,29], the swimbladder subsequently became associated with buoyancy control in species that radiated into less hypoxic waters. Although the nature of the transition from buoyancy control to hearing is unknown, we suggest that sound pressure-evoked M-cell activation and predator evasion co-evolved with, and facilitated, the addition of hearing to swimbladder function.

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1. Zottoli, S. J. *J. exp. Biol.* **68**, 243–254 (1977).
2. Eaton, R. C., DiDomenico, R. & Nissanov, J. *J. Neurosci.* **8**, 2758–2768 (1988).
3. Blaxter, J. H. S., Gray, J. A. B. & Denton, E. J. *J. mar. Biol. Ass. U.K.* **61**, 851–869 (1981).
4. Lauder, G. in *Functional Vertebrate Morphology* (eds Hildebrand, M., Bramble, D., Liem, K. & Wake, D.) 210–229 (Belknap, Cambridge, Massachusetts, 1985).
5. Webb, P. W. *Can. J. Fish Aquat. Sci.* **43**, 763–771 (1986).
6. Nissanov, J. & Eaton, R. C. *Am. Zool.* **29**, 103–121 (1989).
7. Fay, R. *Science* **225**, 951–954 (1984).
8. Sand, O. & Enger, P. S. *J. exp. Biol.* **59**, 405–414 (1973).
9. Fay, R. R. & Popper, A. N. *J. exp. Biol.* **62**, 379–387 (1975).
10. Fay, R. R. in *Hearing and Sound Communication in Fishes* (eds Tavolga, W. N., Popper, A. N. & Fay, R. R.) 189–221 (Springer-Verlag, New York, 1981).
11. Sand, O. & Hawkins, A. D. *J. exp. Biol.* **59**, 797–820 (1973).
12. Popper, A. N. in *Fish Neurobiology* Vol. 1 (eds Northcutt, R. G. & Davis, R. E.) 125–178 (Michigan University Press, Ann Arbor, 1983).
13. Furukawa, T. & Ishii, Y. *Jap. J. Physiol.* **17**, 572–588 (1967).
14. Fay, R. R. & Popper, A. N. *J. exp. Biol.* **61**, 243–260 (1974).
15. Diamond, J. in *Fish Physiology* Vol. 5 (eds Hoar, W. S. & Randall, D. J.) 265–346 (Academic, New York, 1971).
16. Blaxter, J. H. S. & Hoss, D. E. *J. mar. Biol. Ass. U.K.* **61**, 871–879 (1981).
17. Blaxter, J. H. S. & Fuiman, L. A. in *The Mechanosensory Lateral Line: Neurobiology and Evolution* (eds Coombs, S., Görner, P. & Münz, H.) 481–499 (Springer-Verlag, New York, 1989).
18. Eaton, R. C., Nissanov, J. & Wieland, C. M. *J. comp. Physiol. A* **155**, 813–820 (1984).
19. Korn, H. & Faber, D. S. *Brain Res.* **93**, 342–348 (1975).
20. Fay, R. R. & Popper, A. N. in *Comparative Studies of Hearing in Vertebrates* (eds Popper, A. N. & Fay, R. R.) 3–42 (Springer-Verlag, New York, 1980).
21. Chapman, C. J. & Sand, O. *Comp. Biochem. Physiol.* **47A**, 371–385 (1974).
22. Faber, D. S., Fetcho, J. R. & Korn, H. *Ann. N.Y. Acad. Sci.* **563**, 11–33 (1989).
23. Furukawa, T. & Furshpan, E. J. *J. Neurophysiol.* **26**, 140–176 (1963).
24. Moulton, J. M. & Dixon, R. H. in *Marine Bio-Acoustics* Vol. 2 (ed. Tavolga, W. N.) 187–232 (Pergamon, Oxford, 1967).
25. Eaton, R. C. & Kimmel, C. B. *J. comp. Physiol. A* **140**, 337–342 (1980).
26. Fink, S. V. & Fink, W. L. *Zool. J. Linn. Soc.* **72**, 297–353 (1981).
27. Zottoli, S. J. in *Neurobiology of the Mauthner Cell* (eds Faber, D. S. & Korn, H.) 13–45 (Raven, New York, 1978).
28. Tavolga, W. N. in *Fish Physiology* Vol. 5 (eds Hoar, W. S. & Randall, D. J.) 135–205 (Academic, New York, 1971).
29. Liem, K. F. *Am. Zool.* **28**, 739–759 (1988).
30. Enger, P. in *Lateral Line Detectors* (ed. Cahn, P.) 239–248 (Indiana University Press, Bloomington, 1967).
31. Faber, D. S., Korn, H. & Lin, J-W. *Brain, Behav. Evol.* (In the press).

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Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor

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NERVE growth factor plays an important part in neuron–target interactions in the late embryonic and adult brain. We now report that this growth factor controls the proliferation of neuronal precursors in a defined culture system of cells derived from the early embryonic brain. Neuronal precursor cells were identified by expression of the intermediate filament protein nestin. These cells proliferate in response to nerve growth factor but only after they have been exposed to basic fibroblast growth factor. On withdrawal of nerve growth factor, the proliferative cells differentiate into neurons. Thus, in combination with other growth factors, nerve growth factor regulates the proliferation and terminal differentiation of neuroepithelial stem cells.

Cell marking experiments in vertebrates[1–7] and genetic studies in *Drosophila*[8,9] have shown that extrinsic signals regulate the transition of a multipotential precursor to post-mitotic neurons of different types (for review see ref. 10). To identify these signals, embryonic rat striatum primordia were dissected at the time when neurogenesis begins[11] and cultured in serum-free medium. Immediately after plating, more than 95% of rat striatal cells dissociated from embryonic day 13.5–14.5 expressed nestin, an intermediate filament protein specifically found in neuroepithelial stem cells[12,13]. After 2 days *in vitro* most cells were still nestin positive (nestin$^+$) (Fig. 1*a*, dashed line) whereas the 200K subunit (relative molecular mass 200,000) of neurofilament was detectable in a smaller number of cells (Fig. 1*a*, solid line). After 9 days *in vitro*, the cells differentiated into neurons (Fig. 1*a*). These results confirm previously published data that show that cells dissociated from embryonic striatum can differentiate into neurons[14].

The receptor for nerve growth factor (NGF-R) has been localized in many cells throughout the embryonic striatum from embryonic day 14 onward[15]. When, however, NGF was added to dissociated cultures of embryonic striatum, there was no effect on the proliferation, survival or differentiation of this population of nestin$^+$ cells (Fig. 1*b*). Recent experiments suggest that multiple interactions between growth factors occur through the reciprocal regulation of their receptors[16]. We therefore determined whether striatal precursor cells became responsive to NGF after treatment with basic fibroblast growth factor (bFGF).

Recombinant bFGF promoted the proliferation and/or survival of nestin$^+$ cells during 9 days *in vitro* (Fig. 1*c*). The increase in the number of nestin$^+$ cells in the presence of bFGF correlated with the formation of small colonies of nestin$^+$ cells, which became visible after 3–4 days. After 9 days in culture these nestin$^+$ colonies contained no more than 5–10 cells (Fig. 2*a*, *b*). When NGF was added together with bFGF, 17 times more nestin$^+$ cells were found (85% of the cells were nestin$^+$) after 9 days *in vitro* compared with the serum-free medium control experiment and 2.5-times more when compared to bFGF-treated cultures (Fig. 1*d*). With NGF and bFGF, larger colonies of between 25 and 60 cells were seen in the cultures, compared with bFGF alone. All the cells in these colonies were strongly nestin$^+$ (Fig. 2*c*, *d*).

To determine whether the colonies were the product of a single proliferating cell[17], cells were infected with a dilute

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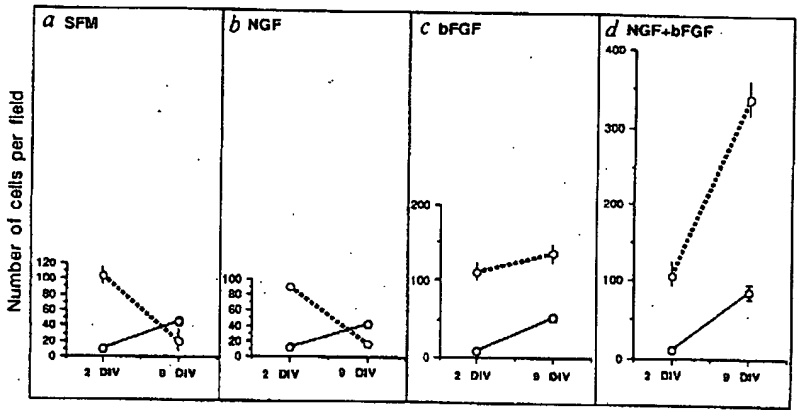

FIG. 1 Effect of NGF and bFGF on the number of nestin$^+$ and neurofilament$^+$ cells. The number of nestin$^+$ (- - - -) and neurofilament$^+$ (——) cells after 2 and 9 days *in vitro* (2 or 9 DIV) is shown for different culture conditions. Rat striata from embryonic days 13.5–14.5 were dissected and transferred into Petri dishes containing Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS) pH 7.2. After removing the meninges the tissue pieces were rinsed in HBSS and pelleted for 5 min, 1,000 r.p.m., 4 °C. A single cell suspension was obtained by gentle mechanical dissociation without the use of trypsin. Cells were counted in a haemocytometer and viability determined by exclusion of trypan blue. Cells (45,000 cm^{-2}) were then plated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) into polyornithine-coated (15 μg ml^{-1}) glass coverslips in 24-well tissue culture plates and cultured at 33 °C in 5% CO_2. 15 h later the cultures were rinsed twice with HBSS and serum-free medium (SFM) (composition 1:1 F12:DMEM including: 5 μg ml^{-1} insulin, 100 μg ml^{-1} transferrin, 20 nM progesterone, 30 nM selenium salt, 60 μM putrescine, 2 mM glutamine, 0.11 mg ml^{-1} sodium pyruvate, 3.7 mg ml^{-1} sodium bicarbonate, 4.3 mg ml^{-1} HEPES buffer) was added. Cells were cultured in SFM supplemented with growth factors where indicated: NGF (150 ng ml^{-1}; Collaborative Research) was replaced every 2 days, recombinant bFGF (5 ng ml^{-1}, Amgen) was applied once at day 1. After 2 and 9 days of culture, the total number of cells was measured by counting 10 fields (0.25 mm^2 each) across the well. Three separate wells were counted for each treatment. After counting, the cultures were fixed in 4% paraformaldehyde in phosphate buffer and immunostained. Following permeabilization with 0.01% Triton, cells were incubated with a 1:1,000 dilution of a rabbit antiserum against nestin (M. Marvin and R. McK.) or with a 1:160 dilution of a mouse monoclonal antibody against the 200K subunit of the neurofilament protein (ICN). After 1 h at room temperature the cells were rinsed in PBS and then incubated for 1 h respectively with a fluorescein-conjugated goat anti-rabbit (1:100, Cappel) or a rhodamine-conjugated goat anti-mouse (1:100, Cappel) secondary antibody. The percentage of immunofluorescent nestin$^+$ (- - - -) and neurofilament$^+$ (——) cells was determined on >400 cells. The data represent the average absolute number ±s.e.m. of nestin$^+$ and neurofilament$^+$ cells counted as described above. The results shown represent one of five independent experiments that reproduced these effects.

suspension of retrovirus expressing the *Escherichia coli lacZ*. Figure 3 shows one isolated colony containing >20 lacZ-positive cells which was expanded for 5 days *in vitro* in the presence of NGF and bFGF following infection after one day *in vitro*. These data show that the formation of colonies was a result of clonal expansion of nestin$^+$ precursor cells.

In Fig. 4 we show that NGF alone can support precursor cell proliferation after a brief exposure to bFGF. The dose–response curve shows that the number of cells was a linear function of NGF concentration in the presence (plain line) or absence (dashed line) of bFGF. The proliferation of striatal cells in response to NGF concentrations of hundreds of nanograms per millilitre may be explained by binding to the low-affinity NGF receptor. The specificity of the proliferative effect of NGF was confirmed by showing that incubating the cells with a monoclonal antibody to NGF completely blocked the increase in cell number. After two days pretreatment with bFGF (5 ng ml^{-1}), the cultures were rinsed and switched either to serum-free medium or to serum-free medium containing NGF (300 ng ml^{-1}) for three days. At this time, a 10-fold molar excess of an anti-NGF antibody (Boehringer) was applied to a set of cultures containing NGF. After six additional days in the presence of the antibody the total number of cells did not increase (166 ± 12) when compared with the cultures not treated with the antibody (439 ± 14). The smaller number of cells in the presence of anti-NGF than in serum-free medium (221 ± 16) might be explained by the secretion of NGF by striatal cells in culture. Furthermore, combination of NGF or bFGF with other growth factors did not produce any effect on cell number (data not shown). The observed effect of NGF may also be indirect, acting through a

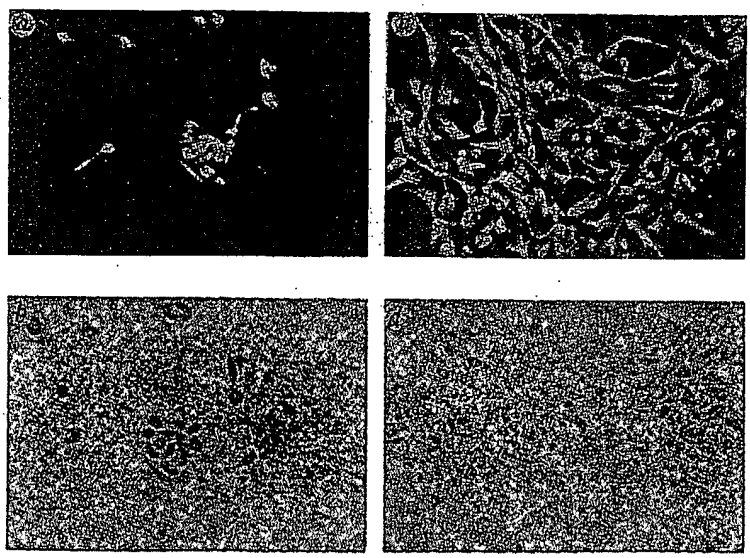

FIG. 2 Colonies of nestin$^+$ cells formed as a consequence of proliferation in the presence of bFGF and NGF. In the presence of bFGF, NGF stimulated the proliferation of colonies of striatal nestin$^+$ cells. After 9 days *in vitro* in bFGF alone (*a*, *b*) or bFGF and NGF (*c*, *d*), cells were immunostained with the anti-nestin antibody as described in the legend to Fig. 1. *a*, *c*, Fluorescent and *b*, *d*, phase-contrast pictures.

FIG. 3 Clonal expansion forms the nestin$^+$ colonies. After 1 day *in vitro*, striatal cultures were infected with a diluted suspension of a retrovirus carrying the *lacZ* gene. The supernatant was previously titred to have 1–3 infected cells in the dish. After 5 days in the presence of NGF (150 ng ml^{-1}) and bFGF (5 ng ml^{-1}), cells were fixed in 0.1% glutaraldehyde, permeabilized with 0.1% Triton X-100 and reacted with 1 mg ml^{-1} X-gal. Isolated clones of *lacZ*$^+$ cells were visible when NGF and bFGF (or NGF after a pretreatment with bFGF) were present in the culture medium. Note that the colony shown contains a clone of blue cells and a few cells which did not express β-galactosidase. In control cultures (no growth factors) 2–3 isolated, single, blue cells were visible in the dish. In an additional control experiment the cultures were fixed after just three days of exposure of the growth factors. In this case there were fewer cells forming the blue colonies.

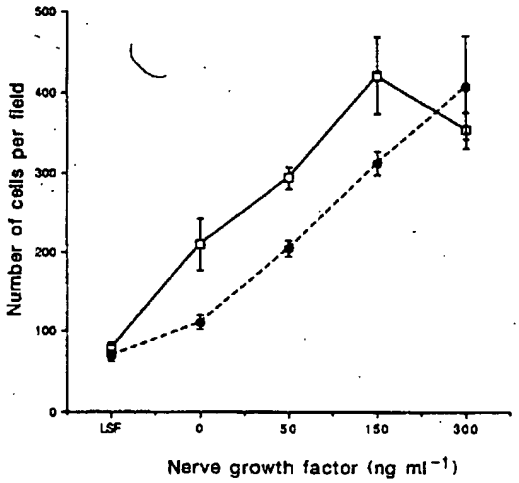

FIG. 4 Influence of NGF concentration on the total number of cells after 9 days *in vitro*. Striatal cultures were prepared as described above and cultured in the presence of various concentrations of NGF for 9 days. Cells were either cultured with NGF and bFGF (5 ng ml^{-1}, ——) or after 2 days of treatment with bFGF the medium was replaced with medium containing NGF alone (- - - -). The total number of cells was scored as described in the legend to Fig. 1 and the results shown are the average cell number ±s.e.m. In the figure, NGF '0 ng ml^{-1}' represents the experimental condition in which bFGF alone is present in the medium during the whole experiment (——) or bFGF for only the two initial days and then replaced with SFM with no NGF (- - - -). During the experiment, NGF was replaced every 48 h. These results represent one of three independent experiments.

subset of cells in the culture which then release some other proliferative factor. Despite these qualifications, the linear dose-response curve and the antibody-blocking experiment show that NGF causes proliferation of nestin$^+$ cells in a completely defined culture medium.

Actively proliferating cells can be identified by their incorporation of the nucleotide analogue 5-bromodeoxyuridine (BrdU). BrdU uptake shows that even after 9 days *in vitro* in the presence of bFGF and NGF many cells were still proliferating. When the growth factors were removed after 9 days *in vitro*, prolifer-

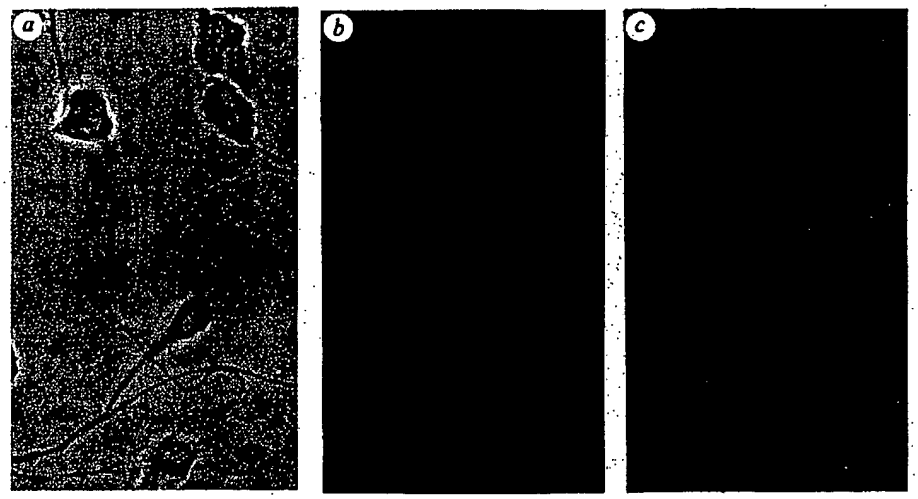

FIG. 5 Proliferating cells differentiated upon removal of growth factors. After 9 days *in vitro* in the presence of bFGF (5 ng ml^{-1}) and NGF (150 ng ml^{-1}) the cultures were shifted to fresh SFM for four additional days. Actively proliferating NGF-responsive nestin$^+$ cells were labelled with 5-bromodeoxyuridine (10 μM BrdU) for 6 h before withdrawal of growth factors. After 18 h in SFM the flat nestin$^+$ cells comprising the colonies started to round up and after 4 days a clear morphological and immunochemical differentiation was evident in the colonies. Immunocytochemistry with anti-BrdU antibody showed that 30% of the cells were labelled (data not shown), indicating that a large proportion of the cultured cells were still actively dividing after 9 days *in vitro*. In growth-factor-free medium the BrdU-labelled cells within colonies assumed small cell bodies with fine processes (*a*), downregulated nestin (*b*) and expressed neurofilament immunoreactivity (*c*). Four BrdU-labelled nuclei can be seen in *a*.

METHODS. After 4 days in SFM, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and processed for triple labelling with anti-BrdU, anti-nestin and anti-neurofilament antibodies. Following denaturation in 1 N HCl and neutralization in 0.1 M sodium borate, cells were incubated for 15 h with an anti-BrdU (Becton-Dickinson 1:10 in 3% goat serum, 0.1% triton in PBS). After rinsing in PBS, a horseradish peroxidase conjugated goat antimouse secondary antibody was applied for 1 h (Biorad 1:30) and visualized with diaminobenzidine (DAB) histochemistry. Nestin and neurofilament staining were performed after the diaminobenzine reaction following the protocol described in the legend to Fig. 1.

ation ceased and the cells went through a clear morphological change, nestin expression was greatly reduced and fine neurofilament positive processes were seen (Fig. 5). These results show that cells which proliferated *in vitro* could still differentiate into neurons.

NGF promotes survival and neurite extension of neurons in the peripheral nervous system of vertebrates[18], but also affects differentiated neurons in the central nervous system *in vitro*[19,20] and *in vivo*[21-24]. Our experiments suggest a developmental role for NGF consistent with the early presence of NGF-R protein[15] and NGF messenger RNA[25] in the striatum. The recent discovery of two closely related growth factors, brain-derived neurotrophic factor and neurotrophin-3 (BDNF and NT-3) suggests that NGF and other members of the NGF family might promote both the proliferation of neuronal precursors and the survival/differentiation of neurons derived from these precursors[26-28]. □

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1. Holt, C. E., Bertsch, T. W., Ellis, H. M. & Harris, W. A. *Neuron* **1**, 15–26 (1988).
2. Luskin, M. B., Pearlman, A. L. & Sanes, J. R. *Neuron* **1**, 635–647 (1988).
3. Price, J. & Thurlow, L. *Development* **104**, 473–482 (1988).
4. Ready, D. F., Hanson, T. E. & Benzer, S. *Devl Biol.* **53**, 217–240 (1975).
5. Turner, D. L. & Cepko, C. L. *Nature* **238**, 131–138 (1987).
6. Walsh, C. & Cepko, C. *Science* **241**, 1342–1345 (1988).
7. Wetts, R. & Fraser, S. E. *Science* **239**, 1142–1145 (1988).
8. Rubin, G. M. *Cell* **87**, 519–520 (1989).
9. Zipursky, S. L. *Trends Neurosci.* **12**, 183–188 (1989).
10. McKay, R. D. G. *Cell* **58**, 815–821 (1989).
11. Bayer, S. A. *Int. J. dev. Neurosci.* **2**, 163–175 (1984).
12. Frederiksen, K. & McKay, R. D. G. *J. Neurosci.* **8**, 1444–1151 (1988).
13. Lendahl, U., Zimmerman, L. & McKay R. D. G. *Cell* **60**, 585–595 (1990).
14. Weiss, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 2238–2242 (1986).
15. Gage, F. H. *et al. Neuron* **2**, 1177–1184 (1989).
16. Birren, S. J. & Anderson, D. J. *Neuron* **4**, 189–201 (1990).
17. Price, J., Turner, D., Cepko, C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 156–160 (1987).
18. Purves, D. *Body and Brain: A Trophic Theory of Neural Connections* (Harvard University Press, Cambridge, Massachusetts, 1988).
19. Hefti, F. & Hartikka, J. *J. Neurosci.* **8**, 2967–2985 (1988).
20. Martinez, H., Dreyfus, C., Jonakait, M. & Black, I. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7777–7781 (1985).
21. Kromer, L. F. *Science* **235**, 214–216 (1988).
22. Williams, L. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 9231–9235 (1986).
23. Hefti, F., Dravid, A. & Hartikka, J. *Brain Res.* **293**, 305–311 (1984).
24. Hefti, F. *J. Neurosci.* **6**, 2155–2162 (1986).
25. Maisonpierre P. C., *et al. Neuron* (in press).
26. Liebrock, J., *et al. Nature* **341**, 149–152 (1989).
27. Maisonpierre, P., *et al. Science* **247**, 1446–1451 (1990).
28. Hohn, A., Liebrock, J., Bailey, K. & Barde, Y.-A. *Nature* **344**, 339–341 (1990).

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Potassium conductances in hippocampal neurons blocked by excitatory amino-acid transmitters

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EXCITATORY amino acids mediate fast synaptic transmission in the central nervous system through the activation of at least three distinct ionotropic receptors: *N*-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-isoxasole-4-propionate (AMPA)/quisqualate (QUIS) and the kainate subtypes (for reviews, see refs 1, 2). They also activate the additional QUIS 'metabotropic' receptor (sensitive to *trans*-1-amino-cyclopentyl-1,3-dicarboxylate, ACPD) linked to inositol phospholipid metabolism[3-5]. We have used hippocampal slice cultures to study the electrophysiological consequences of the metabotropic response. We find that activation of an ACPD-sensitive QUIS receptor produces a 'slow' excitation of CA3 pyramidal cells, resulting from depression of a Ca^{2+}-dependent K^+ current and a voltage-gated K^+ current. Combined voltage-clamp and microfluorometric recordings show that, although these receptors can trigger an increase in intracellular Ca^{2+} concentration[6-8], suppression of K^+ currents is independent of changes in intracellular Ca^{2+}. These effects closely resemble those induced by activating muscarinic acetylcholine receptors in the same neurons and suggest that excitatory amino acids not only act as fast ionotropic transmitters but also as slow neuromodulatory transmitters.

In hippocampal neurons, activation of QUIS 'metabotropic' receptors induces Ca^{2+}-release from intracellular stores[6-8] and reduces voltage-dependent Ca^{2+} currents[9]. All the experiments described here (with the exception of those in Fig. 2*A*) were carried out in the presence of 2–3 mM kynurenate. Similar results were obtained using a combination of D-2-amino-5-amino-5-phosphonovalerate (D-APV, 40 μM) and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX, 20–100 μM). Under these conditions, where the excitatory effects of NMDA, kainate and AMPA were blocked, glutamate still depolarizes CA3 neurons (Fig. 1), markedly increases the number of spikes elicited by a prolonged depolarizing current pulse and blocks the slow afterhyperpolarization (AHP) following spike discharge[10-12]. These effects are reversible, concentration-dependent, do not desensitize with repeated applications and occur in the same concentration range as the ionotropic responses.

In our experiments, carried out in voltage-clamp and in the presence of tetrodotoxin (TTX, 1 μM), the current underlying the AHP was generated by depolarizing voltage steps (50 ms) and seen as a slow outward tail current following the depolarization (Figs 2, 3). To investigate whether the excitation induced by glutamate (Fig. 1) was due to activation of QUIS 'metabotropic' receptors[4-8,13], we compared the effect of QUIS (1 μM) with that of AMPA (1–2 μM) ±2 mM kynurenate (Fig. 2*A*). In control solution (TTX), both AMPA and QUIS induce an inward current, but only QUIS blocks the AHP-current. Kynurenate completely abolishes the inward current produced by AMPA, whereas the effects of QUIS persist ($n = 5$). This effect of low concentrations of QUIS (0.02–1 μM) is only weakly affected by ionotropic receptor antagonists, whereas higher concentrations of QUIS (>1 μM) produce a kynurenate-sensitive component of the inward current. Application of the putative endogenous excitatory amino-acid transmitters (0.25–1 mM) glutamate, aspartate, homocysteate[14] and cysteine[15] ($n = 4$–33) reversibly induce an inward current and reduce or abolish the outward tail current. The results suggest that these novel effects of amino acids[16,17], clearly different from those resulting from activation of kainate receptors[18], were mediated through the activation of non-AMPA/QUIS receptors. These are most probably metabotropic receptors; indeed other metabotropic agonists[19] such as ibotenate (10 μM, $n = 3$) or ACPD (10 μM, $n = 4$) mimic the effects of QUIS.

We found that at least two distinct K^+ conductances are reduced by QUIS. There is considerable evidence that the AHP current blocked by QUIS corresponds to the current which had been termed I_{AHP} (ref. 20). First, its reversal potential shifts by 22 mV, from -100 ± 4.5 to -78 ± 4.0 mV (±s.e.m.) ($n = 5$) when extracellular K^+ was increased from 2.7 to 8.1 mM, a value which is close to the 27-mV shift expected from the Nernst

EXHIBIT B

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Insulinotropic Hormone Glucagon-Like Peptide-1 Differentiation of Human Pancreatic Islet-Derived Progenitor Cells into Insulin-Producing Cells

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Glucagon-like peptide-1 (GLP-1) is an intestinal incretin hormone, derived from the processing of proglucagon, that exerts insulinotropic actions on insulin-producing pancreatic islet β-cells. Recently GLP-1 was shown to stimulate the growth and differentiation (neogenesis) of β-cells and appears to do so by inducing the expression of the homeodomain protein IDX-1 (islet duodenum homeobox-1; also known as PDX-1, pancreatic and duodenal homeobox gene; and as IPF-1, insulin promoter factor), which is required for pancreas development and the expression of β-cell-specific genes. Earlier we identified multipotential progenitor cells in the islet and ducts of the pancreas, termed nestin-positive islet-derived progenitor cells (NIPs). Here we report the expression of functional GLP-1 receptors on NIPs and that GLP-1 stimulates the differentiation of NIPs into insulin-producing cells. Furthermore, confluent NIP cultures express the proglucagon gene and secrete GLP-1. These findings suggest a model of islet development in which pancreatic progenitor cells express both GLP-1 receptors and proglucagon with the formation of GLP-1. Locally produced GLP-1 may act as an autocrine/paracrine developmental morphogen on receptors on NIPs, resulting in the activation of IDX-1 and the expression of the proinsulin gene conferring a β-cell phenotype. GLP-1 may be an important morphogen both for the embryonic development of the pancreas and for the neogenesis of β-cells in the islets of the adult pancreas. (*Endocrinology* 143: 3152–3161, 2002)

THE PREVALENCE of diabetes mellitus is increasing throughout the world. Diabetes is caused to a large extent by a reduction in the fully functioning mass of insulin-producing β-cells that reside within the islets of Langerhans in the pancreas. As a consequent of a reduced mass of pancreatic β-cells, the amounts of insulin produced are insufficient to meet the body's needs, and hyperglycemia ensues (1, 2). Although recent studies indicate that islet transplantation may be a cure for diabetes (3), the availability of pancreata as a source for islet transplantation is severely limited. Therefore, it will be necessary to develop alternative sources of islet tissue. One such source may be progenitor cells that can be expanded *ex vivo*, differentiated into islet tissue, and transplanted.

The glucagon gene encodes a multifunctional proglucagon that is differentially processed by prohormone convertases 1 and 2 in the pancreas and the intestine. In the α-cells of the pancreas, the major product of proglucagon processing is glucagon, although small amounts of glucagon-like peptide-1 (GLP-1) are produced, whereas in intestinal L cells the major proglucagon-derived products are GLP-1 and GLP-2 (4, 5). However, in streptozotocin-induced diabetic rats there is a robust increase in pancreatic prohormone convertases 1 and 2, resulting in a 2-fold increase in the ratio of amidated GLP-1 to total glucagon immunoreactivity (6), indicating that GLP-1 may play a role in regeneration of β-cell mass in a diabetic animal model. GLP-1 binds to specific G protein-coupled receptors on pancreatic β-cells to stimulate insulin secretion via cAMP-dependent pathways (4, 5). When administered to diabetic mice, GLP-1 lowers blood glucose levels and stimulates insulin secretion (7–10). In addition, GLP-1 increases β-cell mass by inducing the differentiation and neogenesis of ductal progenitor cells into islet endocrine cells (8–12). The antidiabetogenic potential of GLP-1 is currently under investigation and shows promise as a therapeutic agent in the treatment of type 2 diabetes (13).

Recently, we identified a distinct population of cells in pancreatic islets and ducts that expresses nestin (14). These nestin-positive islet-derived progenitor cells (NIPs), isolated from adult pancreatic islets, can differentiate in culture into cells with pancreatic exocrine, endocrine, and hepatic phenotypes. We hypothesized that GLP-1 receptors (GLP-1R) must be present on NIPs and that binding of GLP-1 to its receptors on these cells results in activation of the transcription factor IDX-1, a master regulator of endocrine pancreas development (15, 16). IDX-1 then activates the expression of the insulin gene, resulting in a β-cell phenotype (8, 12).

Here we show the expression of GLP-1 receptors on NIPs and that GLP-1 functionally activates NIPs by virtue of their depolarization and resultant increase in intracellular calcium. Notably, the activation of NIPs by GLP-1 is paradoxically inhibited in conditions of high (20 mM) ambient glucose concentrations, unlike the direct glucose-dependent activation of β-cells by GLP-1. Further, we show that GLP-1 stimulates the differentiation of NIPs into a pancreatic en-

Abbreviations: bFGF, Basic fibroblast growth factor; $[Ca^{2+}]_i$, intracellular calcium; EGF, epidermal growth factor; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; INS-LUC, insulin promoter-luciferase construct; IPF-1, insulin promoter factor-1; NIP, nestin-positive islet-derived progenitor cell.

docrine phenotype that expresses the homeodomain protein IDX-1 and the hormones insulin, glucagon, and GLP-1.

Materials and Methods

Reagents

GLP-1-(7–36)amide was obtained from Sigma (St. Louis, MO). Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), leukemia inhibitory factor were obtained from Sigma and Chemicon International (Temecula, CA), respectively. B-27 supplement was obtained from Life Technologies, Inc. (Gaithersburg, MD).

Isolation and culture of NIPs

Human islet tissue was obtained from the Juvenile Diabetes Research Foundation Center for Islet Transplantation, Harvard Medical School (Boston, MA), and the Diabetes Research Institute, University of Miami School of Medicine (Miami, FL). NIPs were isolated as described previously (14). Briefly, islets were washed and cultured in RPMI 1640 medium containing serum, 11.1 mM glucose, antibiotics, sodium pyruvate, β-mercaptoethanol, and growth factors. Within several days, nestin-positive cells grew out from islets. These cells were cloned and expanded in medium containing 20 ng/ml each of bFGF and EGF. In some instances, long-term passaged cells were maintained in 1000 U recombinant human leukemia inhibitory factor. For differentiation, NIPs were incubated with GLP-1 in the absence of serum, and fresh GLP-1 was added every 48 h without changing the medium. In some experiments differentiation was achieved by culturing NIPs in cell culture medium containing B-27 [DMEM/F-12 (1:1), B-27, bFGF, EGF, and antibiotics] as described by Toma *et al.* (17) for the culture of skin-derived precursors and for the differentiation of mouse embryonic stem cells (18). Similar to the skin precursors (17), NIPs cultured in B-27 medium generated spherical clusters of cells that were collected, centrifuged, and replated onto laminin-coated 48-well plates [BD Biosciences, Bedford, MA] and cultured in the B-27-supplemented medium now containing 10 nM GLP-1 and no other added growth factors, *i.e.* bFGF and EGF were absent.

Antibodies

We used rabbit polyclonal antisera to rat IDX-1 (14) and rat GLP-1 receptor (19), which cross-reacts with its human counterpart. The rabbit antihuman nestin was a gift from Dr. C. Messam (NINDS, NIH, Bethesda, MD). Guinea pig antiinsulin and antiglucagon sera were obtained from Linco Research, Inc. (St. Charles, MO). Cy-3- and Cy-2-labeled secondary antisera were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Immunocytochemistry

Cells cultured on Lab-Tek chamber slides (Nunc, Naperville, IL) or gridded coverslips (Bellco Glass, Inc., Vineland, NJ) were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After several rinses in PBS, cells were permeabilized with methanol/Triton-X in some instances, blocked with normal donkey serum for 30 min, and incubated with primary antiserum or preimmune sermum at 4 C. The following day, cells were rinsed with PBS and incubated with secondary antisera (donkey antirabbit and donkey antiguinea pig) labeled with Cy-3 or Cy-2 for 1 h at room temperature. After several washes, coverslips containing cells were mounted onto slides in mounting medium (Vector Laboratories, Inc., Burlingame, CA). Fluorescence images were obtained using a Carl Zeiss (New York, NY) epifluorescence microscope equipped with an Optronics TEC-470 CCD camera (Optronics Engineering, Goleta, CA) interfaced with a Powermac 7100. IP lab Spectrum software (Signal Analytics, Vienna, VA) was used to acquire and analyze images.

RT-PCR

Total cellular RNA prepared from NIP cultures or human islets were reverse transcribed and amplified by PCR for 40–45 cycles as described previously (20). Oligonucleotides for the PCR were as follows: human GLP-1 receptor: forward, 5′-gtgtggcggccaattactac-3′; reverse, 5′-cttggcaagtctgcatttga-3′; and human glucagon: forward, 5′-atctggactccaggcgtgcc-3′; reverse, 5′-agcaatggattccttggcag-3′. An RT-negative control was run for most samples. PCR cycling for glucagon was at 94 C for 1 min, followed by 94 C for 10 sec, 56 C for 10 sec, and 72 C for 1 min (40 cycles), followed by 72 C for 2 min. A hot start PCR was performed for human GLP-1 receptor (GLP-1R) as follows: 94.5 C for 5 min, followed by addition of Taq polymerase and subsequent cycling at 94 C for 10 sec, 54 C for 10 sec, and 72 C for 10 sec (45 cycles). Primer extension at 72 C was performed for an additional 2 min.

Intracellular calcium ($[Ca^{2+}]_i$) measurements

NIPs plated on gridded coverslips were loaded with fura-2 by incubation in standard extracellular saline (138 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES) containing 5.6 mM glucose and supplemented with 2% fetal bovine serum, 0.01% pluronic F-127, and 5 μM fura-2/AM. Cells were loaded for 90 min at room temperature, washed with standard extracellular saline, and then transferred to a Peltier temperature-controlled stage at 32 C. Human serum albumin (0.05%) was added as a carrier protein during experiments with GLP-1-(7–36)amide and Exendin-(9–39), a specific antagonist of GLP-1. Calcium measurements were taken at 0.25 Hz using an IonOptix (Milton, MA) imaging system. The grid location was noted, and fluorescence images of the cells were recorded for subsequent identification of the cells for immunohistochemical staining. The solution in the recording chamber was exchanged by a gravity-fed perfusion system.

RIA

Insulin levels in culture media were measured by an ultrasensitive RIA kit purchased from Linco Research, Inc. and Diagnostic Products (Los Angeles, CA). The detection level for the insulin assay was 8 pg/ml. GLP-1 levels in culture media were measured by a GLP-1-specific RIA that uses rabbit antiserum raised against the C terminus of GLP-1-(7–36)amide and does not cross-react with glucagon or proglucagon.

Transfections

A fragment of the rat insulin I gene promoter that spans nucleotides −410 to 49 bp was fused to the coding sequence of luciferase in the pxp2 basic vector to generate the insulin promoter-luciferase construct (INS-LUC) (21). The human insulin promoter factor-1 (IPF-1) cDNA was a gift from Henk-Jan Anstoot (Sophia Children's Hospital, Rotterdam, The Netherlands). This cDNA was transferred to a cytomegalovirus 5 promoter vector in our laboratory (22). The rat IDX cDNA was cloned previously in our laboratory (23). Adherent NIP cultures plated in 12-well dishes were transfected with 0.6 μg rat INS-LUC and/or 0.125 μg rat IDX-1 cDNA/well for 5 h in serum-free culture medium using Lipofectamine 2000 (2.5 μl/well; Life Technologies, Inc.). A filler plasmid DNA was used to bring the DNA concentration to 1 μg/well. Then, cells were exposed to test substances in medium supplemented with 10% serum. After 20–24 h, cells were lysed, and luciferase activity was measured using a luciferase assay kit (Promega Corp., Madison, WI) in a luminometer (Wallac, Inc., Gaithersburg, MD). These experiments were carried out in duplicate wells and repeated at least three times. In other instances, NIP cultures were plated onto 4-well Lab-Tek chamber slides and transfected with human IPF-1 cDNA (0.2 μg/well) using Geneporter (Life Technologies, Inc.). The following day, transfected cells were incubated with GLP-1 (1–10 nM) in serum-supplemented medium. After 3–4 d, cells were fixed with 4% paraformaldehyde and subjected to immunostaining for IDX-1 and insulin.

Western immunoblot

NIP cultures plated in 10-cm dishes were either transfected with human IPF-1 cDNA using Geneporter or were left untransfected. These cells were subsequently treated with or without 10 nM GLP-1 in serum-supplemented medium for 3–4 d. Then, nuclear extracts were prepared according to the Schreiber method (24), and equal amounts of proteins (20 μg) were loaded and electrophoresed on premade NuPAGE (Invitrogen, Carlsbad, CA) gels according to the manufacturer's recommendations. The proteins were transferred onto a nitrocellulose mem-

brane and subjected to an IPF-1 immunoblot procedure as described previously (23).

Results

GLP-1 receptor expression in NIPs

We examined human NIPs for the presence of GLP-1R by immunocytochemistry. Receptor immunoreactivity was detected in the majority of NIPs (>60%; Fig. 1A). To further confirm the immunocytochemical identification of GLP-1R in NIPs, we performed an RT-PCR of GLP-1 receptor mRNA prepared from NIP cells and detected the product of the correct size (346 bp) for the GLP-1R (Fig. 1B). Clonal variation in the relative amounts of GLP-1R mRNA between lines was seen; receptor expression was lower in some NIP clones than others (see clone 9 *vs.* 2 in Fig. 1B), but was undetectable in only a minority of clones. The expression of GLP-1 receptors in NIPs indicates the potential for GLP-1-mediated regulation of islet progenitor cell differentiation by GLP-1.

Functional GLP-1 receptor signaling in NIPs

The application of GLP-1 -(7–36)amide to single isolated NIPs elevates $[Ca^{2+}]_i$. Cells were plated onto gridded coverslips to permit subsequent immunohistochemical staining of the same cells to test for nestin expression. All cells examined that increased $[Ca^{2+}]_i$ in response to GLP-1 were nestin positive. In contrast to β-cells prepared from adult human islets, in human NIPs, GLP-1 stimulated $[Ca^{2+}]_i$ at basal (5.6 mM; n = 45 cells) glucose, but had no effect on $[Ca^{2+}]_i$ in the presence of high (20 mM) glucose (Fig. 2A). These glucose-related effects on $[Ca^{2+}]_i$ responses in NIPs were reproduced by forskolin (Fig. 2B), suggesting that the effects of GLP-1 on NIPs are mediated via the activation of G_s and cAMP production, the same signaling pathway used by GLP-1 in adult islet-derived β-cells. However, the glucose dependence of GLP-1 in NIPs differs strikingly from that in adult β-cells, inasmuch as in adult β-cells the actions of GLP-1 are markedly directly dependent on glucose concentrations (25, 26). These findings suggest that the coupling of glucose signaling with cAMP signaling (25) in NIP progenitor cells is different from that in adult islet-derived β-cells. The pretreatment of single isolated NIPs with the peptide exendin-(9–39), a specific antagonist of GLP-1, prevents the increase in $[Ca^{2+}]_i$ mediated by GLP-1 (Fig. 2, C and D). These inhibitory effects of the GLP-1R antagonist exendin-(9–39) on $[Ca^{2+}]_i$ responses suggest that the same isoform of GLP-1R is expressed in NIPs as that expressed in β-cells. The increase in $[Ca^{2+}]_i$ mediated by GLP-1 on NIPs was inhibited by extracellular La^{3+} (5 μM), indicating that GLP-1 is activating $[Ca^{2+}]_i$ influx, consistent with its known role to depolarize β-cells (Fig. 2E). We demonstrate further that tolbutamide (100 μM) stimulates the $[Ca^{2+}]_i$ elevation in NIPs, indicating that NIPs must also express ATP-sensitive K^+ channels (Fig. 2F). These findings suggest that GLP-1 induces membrane depolarization and activation of voltage-dependent Ca^{2+} channels in NIPs, consistent with its known mechanism of action in β-cells. However, unlike its known actions in β-cells, the activation of ion channels in NIPs is inhibited by high (20 mM) glucose.

GLP-1 induces differentiation of NIPs into insulin-secreting cells

Previous studies demonstrated the insulinotropic actions of GLP-1 as well as its ability to stimulate β-cell neogenesis in partial pancreatectomized rats (9). Therefore, we determined whether GLP-1 would induce differentiation of human NIPs into insulin-secreting cells. As described previously (14), human islets were cultured in medium containing bFGF and EGF for 14 d. The majority of islets became degranulated, and a monolayer of cells grew out from them. Immunocytochemical analysis of such islet cultures showed that the outgrowing cells were mostly all nestin positive and insulin negative; just a few cells in the monolayer expressed insulin (Fig. 3A). NIPs were picked from these cultures and expanded in growth factor-supplemented medium (passage 1) for 3 d as described previously (14). In certain instances

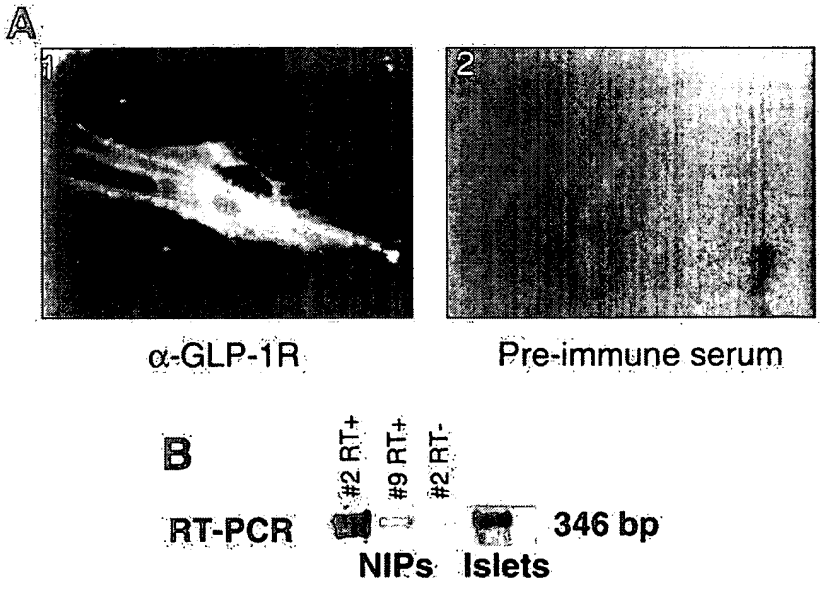

FIG. 1. Expression of GLP-1R on pancreatic islet-derived stem/progenitor cells. A, NIPs (passages 6–8) plated on gridded coverslips were fixed and subjected to immunocytochemical detection with antiserum to GLP-1R (α-GLP-1R) (Cy-3, rendered intense *white* on modifed photomicrograph) or a preimmune serum control. Note the punctate fluorescence on the surface of the cell, typical of receptor aggregation. B, RT-PCR of RNA prepared from different clones of NIPs (passages 4–8) using oligonucleotide amplimers to human GLP-1R give the predicted 346-bp product, which was confirmed by Southern blotting. Human islet tissue was used as the positive control.

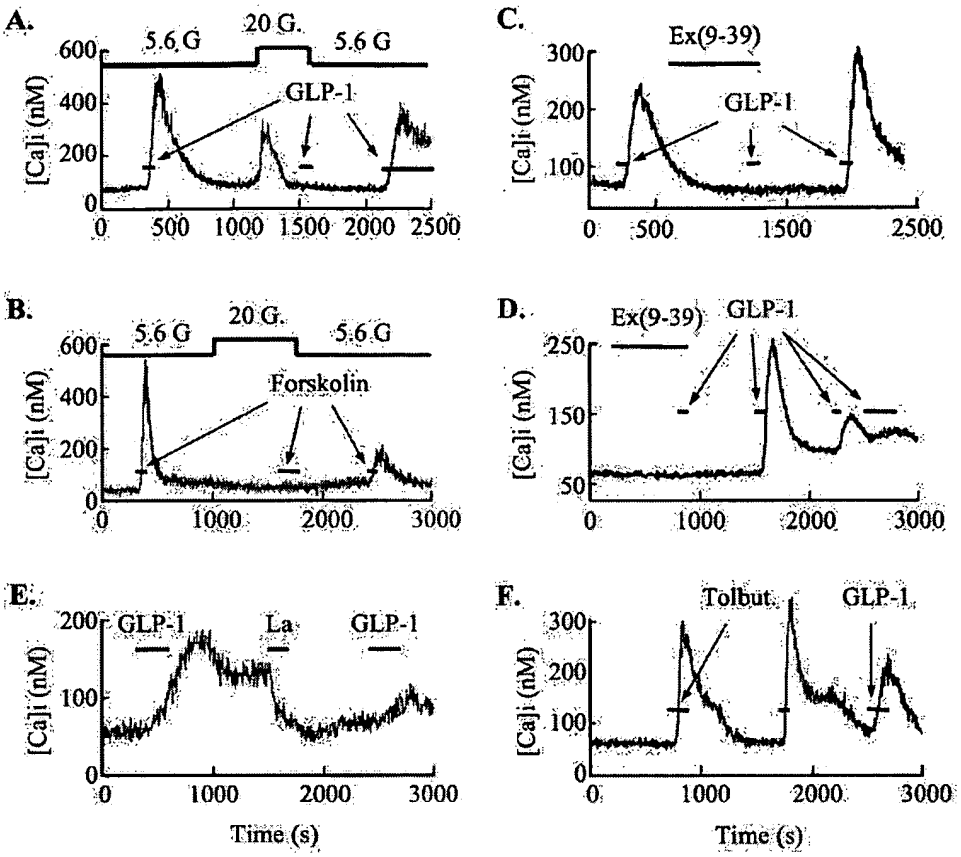

FIG. 2. GLP-1-(7–36)amide and tolbutamide stimulate $[Ca^{2+}]_i$ influx in NIPs. A, Fura-2-loaded cells bathed in 5.6 mM glucose show an increase in the $[Ca^{2+}]_i$ response to 10 nM GLP-1. Increasing extracellular glucose to 20 mM (20 G) also caused an increase in $[Ca^{2+}]_i$, but application of GLP-1 in 20 mM glucose failed to produce an additional $[Ca^{2+}]_i$ response. A third application of GLP-1 on returning to 5.6 mM glucose produced a $[Ca^{2+}]_i$ response. These effects of GLP-1 on $[Ca^{2+}]_i$ were observed in 45 different cells tested. B, The glucose-dependent effects of GLP-1 were reproduced by 10 mM forskolin, suggesting that the $[Ca^{2+}]_i$ elevation is mediated by the cellular cAMP level. C, The GLP-1-mediated increase in $[Ca^{2+}]_i$ was reversibly inhibited by 10 nM exendin-(9–39). This inhibitory effect was not due to receptor desensitization (D), because application of GLP-1 in the presence of the GLP-1 receptor antagonist exendin-(9–39) failed to produce a response, whereas subsequent applications of GLP-1 after washout of exendin-(9–39) produced repeated $[Ca^{2+}]_i$ elevations. E, The GLP-1-mediated increase in $[Ca^{2+}]_i$ is inhibited by 0.5 mM extracellular La^{3+}, an inhibitor of Ca^{2+} influx, suggesting that GLP-1 stimulates Ca^{2+} influx. F, NIPs bathed in 5.6 mM glucose were stimulated with 100 mM tolbutamide (Tolbut.) and responded to repeated applications of tolbutamide with increases in $[Ca^{2+}]_i$. Application of 10 nM GLP-1 also stimulated an increase in $[Ca^{2+}]_i$, suggesting that GLP-1 acts by depolarizing the cells. Shown in each panel are representative recordings from single NIPs (clone 006a) that respond to the above-mentioned test substances.

NIPs that were expanded for 3–5 d spontaneously expressed insulin. We find that at this stage of passage (30–40 cells/dish) the vast majority of NIPs were nestin positive and insulin negative (Fig. 3B). When NIP cultures were expanded for 7–12 d and then treated with GLP-1, a subset of cells became insulin positive (Cy-2; *green*) and nestin negative (Cy-3; *red*; Fig. 3, C and D). Incubation with exendin-(9–39), a specific antagonist of GLP-1, abolished the appearance of immunostaining for insulin (Fig. 3E). The cells treated with GLP-1 also changed their morphology, becoming more rounded and flattened (Fig. 3D *vs.* Fig. 3, C and E). The percentage of differentiated cell progeny by virtue of insulin staining varies from 5% or less to 30% depending on the particular cloned NIP culture tested. Treatment with exendin-4, a long-acting analog of GLP-1, induced a 2- to 3-fold increase in insulin secretion, as measured by RIA of the cell culture medium (Fig. 3G). The secretory response to exendin-4 was detected in 30% of the NIP clones tested. In some culture wells confluence alone was sufficient to initiate the secretion of small amounts of insulin, indicating that cell confluence can induce differentiation to some extent.

Human NIPs that have been repeatedly passaged lose their ability to secrete insulin in response to GLP-1. However, a modification of the differentiation protocols of these cells can render them again responsive to GLP-1. In these modified experiments, long-term passaged NIPs (≤6 months) were cultured in medium supplemented with B-27, bFGF, and EGF for 4–6 d or more. Similar to skin-derived precursor stem cells (17), NIPs in this medium form floating clusters after 6 d of culture (Fig. 4A). For differentiation experiments, these clusters were collected, centrifuged, and plated on laminin-coated dishes in the B-27-supplemented medium now containing 10 nM GLP-1 but no growth factors (bFGF and EGF). A week later, cells grew out from the clusters, which were then fixed and subjected to immunocytochemistry for insulin and IDX-1.

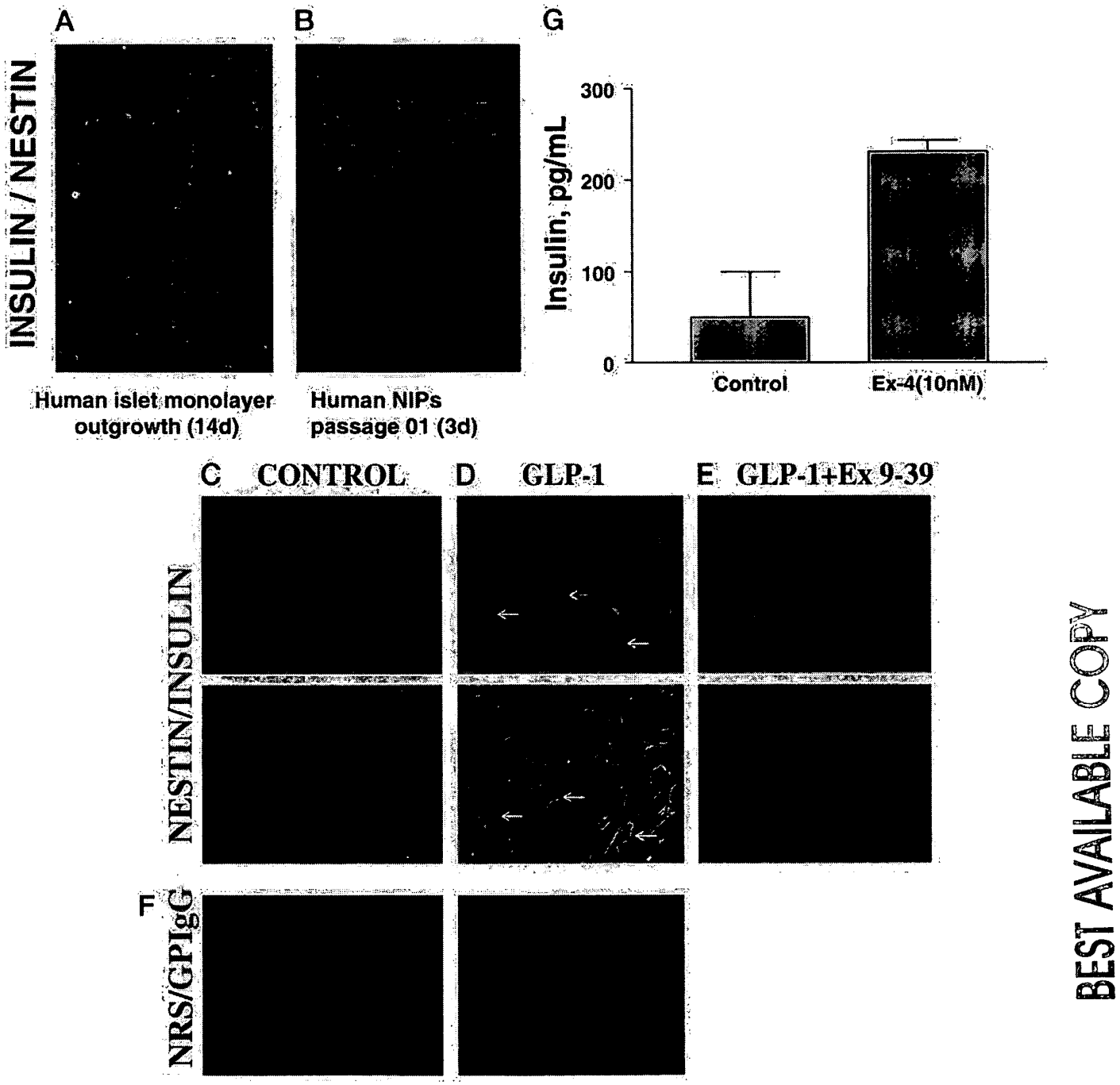

FIG. 3. GLP-1 induces differentiation of NIPs into insulin-producing cells. A, Human islets were cultured in growth medium containing bFGF and EGF for 14–18 d. The monolayer of cells that grew out from the islet was fixed with 4% formaldehyde and immunostained with antiserum for insulin (Cy-2, *green*) and nestin (Cy-3, *red*). The majority of cells that grew out from islets were nestin positive and insulin negative. B, NIPs that grew out from islets were picked, replated, expanded in the same medium (passage 1) for 3–5 d, fixed, and immunostained for nestin (*red*) and insulin (*green*). Most cells at this stage were nestin positive and insulin negative. C, Differentiation of NIP cultures treated with GLP-1. NIP cultures (clone 016f, passage 1) were expanded for 7–12 d. Between d 10 and 12, cultures were replenished with serum-free medium alone (control, C) or containing 10 nM GLP-1-(7–36)amide or GLP-1 plus exendin-(9–39) (100 nM; Ex9–39), a specific antagonist of GLP-1 (D and E, respectively). Seventy-two to 96 h later, cells were fixed and immunostained for nestin (Cy-3, *red*) and insulin (Cy-2, *green*). A subset of cells became nestin negative and insulin positive (indicated by *white arrows*, D). F, To control for background staining, GLP-1-treated cells were incubated with preimmune normal rabbit serum (*red*) and guinea pig IgG (*green*). Note the change in cell morphology when cells were treated with GLP-1 (D and F *vs.* C and E). G, Insulin secretion from NIP cultures treated with exendin-4. NIP cultures (passage 1) were expanded for 7–12 d and treated with 10 nM exendin-4, a GLP-1 agonist, for 48–72 h. Media were collected and assayed for insulin. Values are the mean ± SEM of four wells obtained from two different clones of NIPs.

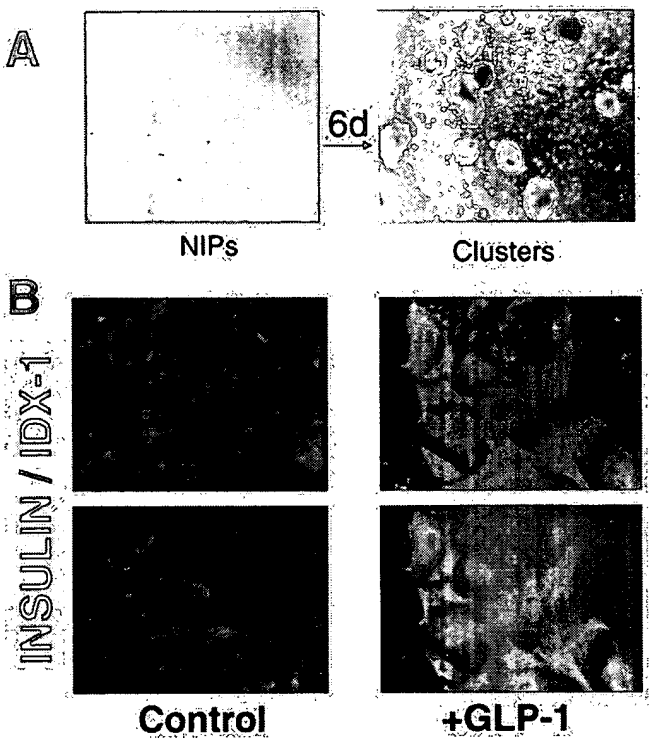

FIG. 4. Differentiation of long-term passaged (≤6 months) NIPs. A, Confluent NIP cultures (n = 2; clone 009b) were trypsinized and plated in B-27-supplemented medium (see *Materials and Methods*). The morphology of cells changed as they become more flattened (panel 1), and by 6 d the cells generated clusters (panel 2). B, The clusters were collected, centrifuged, and replated into laminin-coated 48-well plates in B-27 medium alone (control) or supplemented with 10 nM GLP-1. The cells that grew out from the clusters after 1 wk were then rinsed, fixed, and subjected to immunocytochemical detection of IDX-1 (Cy-3, *red*) and insulin (Cy-2, *green*).

A subset of cells became insulin and IDX-1 positive in the wells treated with GLP-1 as opposed to control cells without treatment with GLP-1 (Fig. 4B).

Transfection and expression of IDX-1 in NIPs

The homeodomain protein IDX-1 is critical for pancreas development (27) and plays a major role in transcriptional regulation of the insulin gene (15). It has been shown that GLP-1 agonists induce the expression of IDX-1 (8, 9) and that the expression of IDX-1 is sufficient by itself to induce the expression of insulin in liver cells (28) and in pancreatic ductal cells (12). We previously reported that IDX-1 is expressed in differentiated early passage NIP cell populations (14). We reconfirmed this observation in the current study using DNA binding assays with nuclear extracts prepared from differentiated confluent NIP cultures. A radioactively labeled, synthetic oligonucleotide probe encompassing the cytosine thymidine-II region of the human insulin promoter sequence formed a distinct complex that was eliminated when extracts were incubated with an antiserum for IDX-1, confirming the authenticity of IDX-1 in these cultures (data not shown). However, in long-term NIP cultures there was a loss/diminution of endogenous IDX-1 levels.

Next, we addressed whether GLP-1-induced differentiation of NIPs into insulin-expressing cells might correlate with the expression of IDX-1. Accordingly, we transiently transfected rat IDX-1 cDNA with a fragment of the rat insulin I promoter sequence conjugated to a luciferase construct (INS-LUC) into long-term (>6 months to 1 yr) NIP cultures and treated them with GLP-1 or forskolin. As shown in Fig. 5, reexpression of IDX-1 increased basal insulin promoter activity, and this effect was more pronounced when transfected NIPs were treated with GLP-1. In contrast, forskolin enhanced INS-LUC activity regardless of IDX-1 levels, suggesting that the GLP-1 effect on insulin gene expression in NIP cultures may be mediated by increased expression of IDX-1.

We also hypothesized that a certain concentration of IDX-1 in the cells in conjunction with the presence of GLP-1 is required for NIPs to convert into insulin-producing cells. To test this hypothesis, we treated NIPs transfected with human IDX-1 cDNA or untransfected long-term subconfluent NIP cultures (>3 months) with either 10 nM GLP-1 or vehicle for 3–4 d. Later, cultures were either fixed and immunostained with an antibody against human IDX (*red*) and insulin (*green*) or subjected to Western immunoblot analysis for nuclear IDX-1 protein. Immunostaining results show an overall increase in IDX-1 expression levels in transfected cells (four of five times) compared with untransfected cells (Fig. 6, A *vs.* B and C, *upper panels*). These findings were further confirmed by Western immunoblot analyses, which also suggest that GLP-1 treatment increases IDX-1 levels in NIPs transfected with an IDX-1 expression plasmid (Fig. 6D). However, insulin was induced in a subset of transfected and treated NIPs in only two of the above experiments, as shown in a representative experiment in Fig. 6C (*lower panel*) and was not

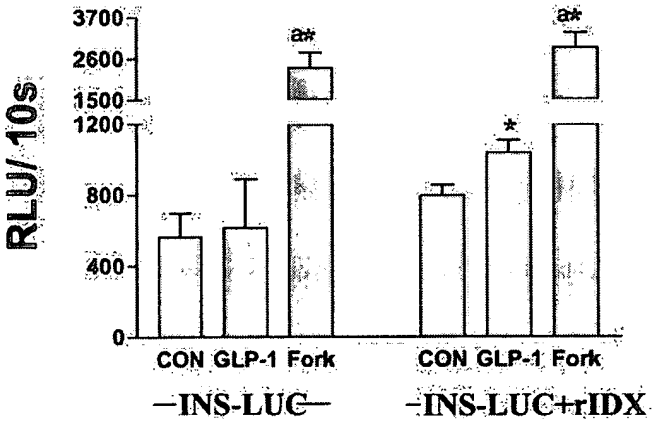

FIG. 5. GLP-1-induced differentiation of NIPs is mediated through a pancreas-specific transcription factor, IDX-1. A, NIP cultures transfected with −410 INS-LUC (INS-LUC) alone (*left*) or also with rat IDX-1 cDNA (*right*) were treated with forskolin (10 μM; Fork), GLP-1 (10 nM), or vehicle (CON) for 20 h. Then cells were lysed and assayed for luciferase activity. Relative light units (RLU) were measured for 10 sec (10s)/sample. Values represent the mean ± SEM of at least four wells from two experiments using clone 06. GLP-1-induced (*, $P < 0.05$) stimulation of INS-LUC activity is IDX-1 dependent, whereas that produced by forskolin (a*) is IDX-1 independent. Note that transfection with empty vectors (pxp2 and cytomegalovirus 5 promoter) yielded background units that were not altered by either treatment (data not shown).

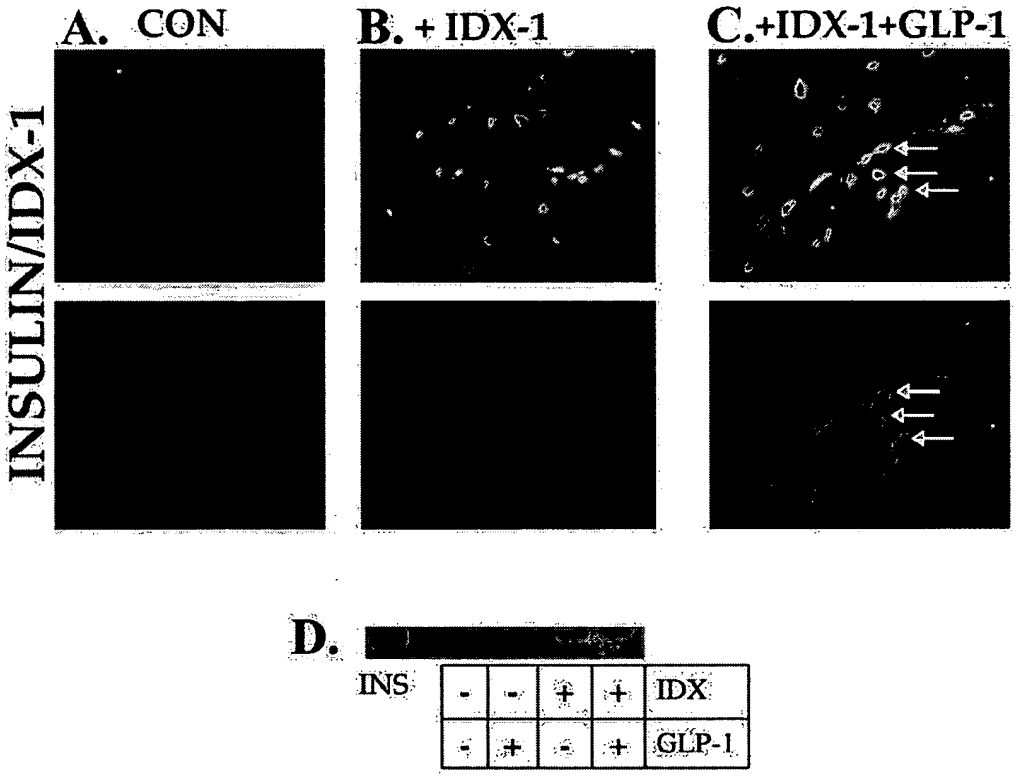

FIG. 6. A–C, Immunocytochemical analysis of insulin expression in NIP cultures transfected with human IDX-1. NIP cultures (passages 6–11) plated on Lab-Tek chamber slides were left untransfected (control (CON; A) or were transfected with human IDX-1 expression plasmid (+IDX-1; B and C). Five hours later, cultures were re-fed with medium containing serum. The following day, NIPs were treated with 10 nM GLP-1 (+IDX-1+GLP-1; C) or vehicle control (B). Three days later, cultures were fixed, permeabilized with methanol/Triton-X, and subjected to dual fluorescence immunocytochemical detection of IDX-1 (Cy-3, *red*) and insulin (Cy-2, *green*). Shown are representative figures in which NIPS (clone 006a) transfected with IDX-1 remains insulin negative/low (B, *lower panel*), and a subset of those treated with GLP-1 stains for insulin (C, *lower panel*). Note that IDX-1 immunostaining is more intense in those cultures that were transfected and treated with GLP-1 than in untransfected, untreated controls (B and C *vs.* A, *upper panels*). D, Western immunoblot analysis of IDX-1 expression levels in response to GLP-1. NIP cultures transfected with human IDX-1 cDNA or left untransfected were treated with GLP-1 (10 nM) or vehicle. Then cells were lysed, and nuclear extracts were prepared. Samples were electrophoresed and immunoblotted with an antibody specific for IDX-1. An extract from a rat insulinoma cell line (INS) was used as a positive control. Note the absence of endogenous IDX-1 in this clone of NIPs (>6 months in culture).

induced in other instances even in the presence of increased IDX-1 expression (Fig. 6B, *lower panel*). Taken together, these experiments suggest that IDX-1 may play a role in GLP-1-induced differentiation of NIPs into insulin-producing cells.

NIPs express the proglucagon gene and secrete GLP-1

Major regulators of expression of the nestin gene in neural stem cells are the POU homeodomain proteins Brn-2 and Brn-4 (29). Brn-4 is known to be a key activator of the expression of the proglucagon gene by interactions with the G_1 element located in the proximal α-cell-specific expression promoter of the proglucagon gene (30). Therefore, we examined NIPs for expression of the proglucagon gene. Although the proglucagon gene is not expressed in nonconfluent NIPs (Zulewski, H., unpublished observations), when NIPs approach confluence and begin to differentiate, they express the proglucagon gene, as shown by RT-PCR (Fig. 7A) and immunocytochemistry (Fig. 7B), and secrete GLP-1 into the culture medium (Table 1). Because NIPs express functional GLP-1Rs, the later expression of GLP-1 by early differentiating NIPs suggests that GLP-1 may function as an autocrine /paracrine morphogen in the differentiation of multipotential NIPs to pancreatic endocrine cells.

Discussion

Our findings demonstrate the presence of functional GLP-1Rs on pancreatic progenitor cells and suggest a direct role for GLP-1 in the differentiation of NIPs into insulin-producing cells. Differentiation occurs in a subset of NIP cells that is induced to produce insulin. GLP-1 exerts diverse effects on β-cells, including stimulation of cAMP formation (25, 31) and activation of phosphoinositol 3-kinase (32–34), which, in turn, activates several downstream signaling targets that stimulate insulin secretion. We characterized the electrophysiological responses of NIPs to GLP-1 and found that NIPs bathed in physiological concentrations of glucose (5.6 mM) show an increase in $[Ca^{2+}]_i$ in response to the application of 10 nM GLP-1. Paradoxically, a higher glucose concentration (20 mM) rendered GLP-1 ineffective in evoking a $[Ca^{2+}]_i$ response. Like GLP-1, the cAMP agonist forskolin also evoked a similar increase in $[Ca^{2+}]_i$ in NIPs at 5.6 mM glucose, and the response to forskolin was abrogated at 20 mM glucose. These results suggest that NIPs

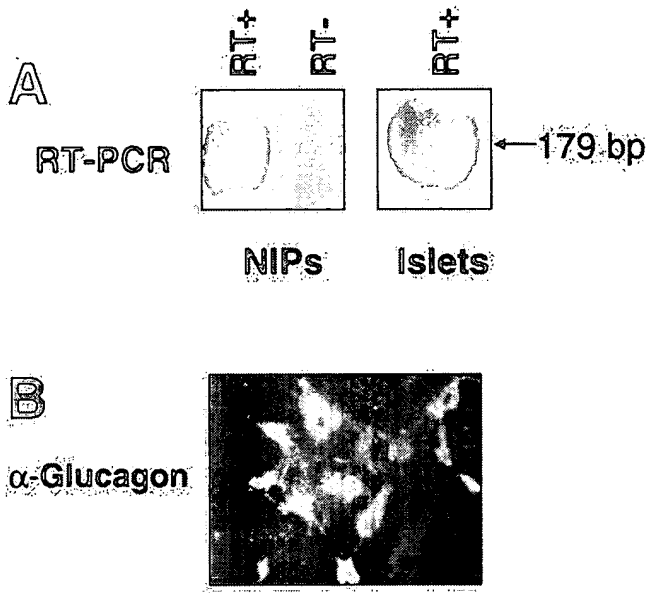

FIG. 7. A, Proglucagon is expressed in confluent NIP cultures. RT-PCR was performed of RNA prepared from NIPs (clone 9, passage 4) using oligonucleotide amplimers to human proglucagon giving the predicted 179-bp product. Islets were used as the positive control. B, NIPs were differentiated by culturing them in B-27-supplemented medium and plating them on wells (as described in *Materials and Methods*). Then cells were rinsed, fixed, and stained with an antiserum to glucagon (α-glucagon; Cy-2; intense *white* on modified figure).

contain a glucose-sensing mechanism. In support of these observations, our previous study demonstrates that Glut-2 mRNA is expressed in NIPs (14). Perhaps GLP-1 receptors are more abundant in conditions of physiological glucose concentrations, but are decreased in high glucose concentrations (35). Indeed, a recent study by Hui *et al.* (12) demonstrates that GLP-1R mRNA in ARIP cells (a pancreatic ductal cell line) is decreased in high glucose concentrations. Similar to islet β-cells, the NIP pancreatic progenitor cells respond to tolbutamide, a drug that binds sulfonylurea receptors on β-cells to depolarize them by closing ATP-sensitive K channels and to stimulate insulin secretion (36, 37). These studies demonstrate that functional sulfonylurea receptors are present on NIP pancreatic progenitor cells. Although our studies show the existence of tolbutamide-responsive ATP-sensitive K^+ channels on NIPs and a depolarizing response to GLP-1, the apparent loss of augmentation of the response in conditions of high glucose (20 mM) is in contrast to the glucose-responsive sensitivity of β-cells to GLP-1 (25, 26). These observations suggest that unlike β-cells that increase their responsiveness to GLP-1 in conditions of acute elevations of glucose concentrations, the response of progenitor cells such as NIPs appears to be impaired in high glucose. It is important to distinguish between long-term glucotoxicity of β-cells in which cellular function is impaired after several days of exposure to high glucose (21, 38) and the acute impairment of NIP responses observed in just a few minutes. This apparent circumstance of the impairment of NIPs to respond to elevated glucose may be relevant to the deleterious effects of elevated blood glucose levels in individuals with diabetes, such that elevated glucose might impair the neogenesis of new β-cells from progenitor

TABLE 1. Secretion of GLP-1 by cultured NIPs

Well no.	GLP-1 (pg/ml)
1	12.7
2	56.7
3	79.8
4	21.2
5	48.1

Medium was collected from NIP cultures (passage 1), RIA was performed to assayed for secreted forms of GLP-1. The GLP-1 RIA is specific for the detection of the processed GLP-1-(7–36) amide and does not detect proglucagon, glucagon, or GLP-2. Shown are the values from five representative wells (clone 11).

precursor cells. Such glucotoxicity-mediated impairment of β-cell neogenesis, is expected to be accompanied by accelerated glucotoxic β-cell apoptosis (39–41).

The endocrine cells of the rat pancreas turn over every 40–50 d by processes of apoptosis and neogenesis (42). Neogenesis refers to the differentiation of new islet cells from progenitor cells residing in islets (14) and ducts (43–45). There have been several reports of the differentiation of pancreatic duct-derived cell lines into insulin-producing cells by growth factors (46), and GLP-1 is also implicated as a differentiation-inducing agent (10, 12). The administration of exendin-4 to rats stimulates β-cell neogenesis, resulting in increased β-cell mass (9). GLP-1 is now being considered as a potential new therapeutic agent for type 2 diabetic patients (13).

In their undifferentiated state, NIPs are nestin positive and IDX-1 and insulin negative. When exposed to GLP-1, a subset of cells became nestin negative and IDX-1 and insulin positive. Accordingly, insulin secretion by RIA was also detected in these cells. The differentiation of serially passaged NIP cultures into insulin-producing cells was accelerated by transfecting IDX-1 into NIPs before treating them with GLP-1. These findings are in agreement with those of Hui *et al.* (12), who also showed that transfection of PANC-1, a ductal cell line with IDX-1, followed by treatment with GLP-1 induced insulin bioynthesis.

The level of IDX-1 in NIPs may be critical; perhaps when NIP cultures are sequentially passaged, the level of endogenous IDX-1 falls, which is then corrected by transfecting in IDX-1. However, transfection with IDX-1 *per se* did not differentiate NIPs into insulin-producing cells. Treatment of IDX-1-transfected cells with GLP-1 was necessary to induce insulin bioynthesis in a subset of NIPs. Perhaps, transfection of IDX-1 into NIPs up-regulates GLP-1R expression, thus making it more responsive to its ligand. This was demonstrated by Hui *et al.* (12) using IDX-1 transfected PANC-1 cells. Consistent with our findings Wang *et al.* (47) demonstrated that the level of IDX-1 expression defines endocrine pancreatic gene expression. Like embryonic stem cells, the clonal variation in NIPs makes these kind of studies challenging in that responses to GLP-1 will depend not only on the presence or absence of its receptor, but also on the level of receptor expression (see Table 2 for frequencies of events).

Proglucagon gene expression appears to be restricted to endocrine pancreas, intestine, and brain (4, 5). The posttranslational processing of proglucagon is different in each of these tissues. Although the major proglucagon-derived peptide hormone in the pancreas is glucagon, GLP-1 is also

TABLE 2. Events/observations

Events/observations	Frequency	Clones tested
1. GLP-1 receptors on NIPs	≤60% (clone 006a)	3/3 clones; 006a, 009b, 002c; some have higher receptor expression than others
2. GLP-1 mediated differentiation of NIPS into insulin-producing cells		
a) Early passage	≤5–30%	3/3 clones; 010d, 011e, 016f
b) Long-term cultures		
Transfecting rIDX4 INS-LUC	≤66%	2/3 clones; 006a, 015g, 005h
Transfecting hIDX and immunostaining for insulin	↑ IDX-1 ≤80% ↑ Insulin ≤40%	3 clones tested; 006a, 013i,009b

produced. It is surprising that the proglucagon gene appears not to be expressed in nonconfluent passage NIPs, but then becomes expressed when the NIPs become confluent and differentiate. Brn-4 is known to activate nestin gene expression (29) and is a critical α-cell-specific activator of the proglucagon gene by interactions on the G_1 enhancer in the proximal promoter of the gene (30). Of note, we recently reported that the experimental misexpression of Brn-4 in the early developing pancreas (mouse embryonic d 8.5–9.5), directed by the IDX-1 promoter in transgenic mice, results in ectopic expression of the proglucagon gene in the later-developing insulin-producing β-cells (48). Because Brn-4 appears to play a critical role in the expression of both the nestin gene characteristic of stem cells (29) and the proglucagon gene, and the expression of Brn-4 at the time of activation of the IDX-1 promoter during development activates proglucagon gene expression (48), it is tempting to speculate that NIPs may be precursors of the IDX-1-expressing epithelial cells that appear in the foregut of the early mouse embryo (embryonic d 8.5–9.5) that is destined to give rise to the pancreas. Further, we speculate that the expression of the GLP-1R in NIPs coupled with the expression of proglucagon and resultant GLP-1 may establish an autocrine/paracrine hormonal feedback loop that is important in instructing the differentiation of NIPs into pancreatic endocrine cell lineages, *e.g.* β-cells. In addition, our findings are consistent with the reported observations that fetal pancreas-derived progenitor cells give rise to endocrine cells that initially express proglucagon (49) and later coexpress proglucagon and proinsulin (50).

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References

1. **Kruszynska YT, Olefsky JM** 1996 Cellular and molecular mechanisms of non-insulin dependent diabetes mellitus. J Invest Med 44:413–428
2. **O'Rahilly S** 1997 Science, medicine, and the future. Non-insulin dependent diabetes mellitus: the gathering storm. Br Med J 314:955–959
3. **Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM, Warnock GL, Larsen I, Shapiro AM** 2001 Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes 50:710–719
4. **Kieffer TJ, Habener JF** 1999 The glucagon-like peptides. Endocr Rev 20: 876–913
5. **Drucker DJ** 1998 Glucagon-like peptides. Diabetes 47:159–169
6. **Nie Y, Nakashima M, Brubaker PL, Li QL, Perfetti R, Jansen E, Zambre Y, Pipeleers D, Friedman TC** 2000 Regulation of pancreatic PC1 and PC2 associated with increased glucagon-like peptide 1 in diabetic rats. J Clin Invest 105:955–965
7. **Greig NH, Holloway HW, De Ore KA, Jani D, Wang Y, Zhou J, Garant MJ, Egan JM** 1999 Once daily injection of exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose concentrations. Diabetologia 42:45–50
8. **Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF, Egan JM** 2000 Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes 49:741–748
9. **Xu G, Stoffers DA, Habener JF, Bonner-Weir S** 1999 Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276
10. **Zhou J, Wang X, Pineyro MA, Egan JM** 1999 Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. Diabetes 48:2358–2366
11. **Habener JF** 2001 Glucagonlike peptide-1 agonist stimulation of β-cell growth and differentiation. Curr Opin Endocrinol Diabetes 8:74–81
12. **Hui H, Wright C, Perfetti R** 2001 Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. Diabetes 50:785–796
13. **Gutniak MK, Svartberg J, Hellstrom PM, Holst JJ, Adner N, Ahren B** 2001 Antidiabetogenic action of glucagon-like peptide-1 related to administration relative to meal intake in subjects with type 2 diabetes. J Intern Med 250:81–87
14. **Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF** 2001 Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50:521–533
15. **Stoffers DA, Thomas MK, Habener JF** 1997 Homeodomain protein IDX-1: a master regulator of pancreas development and insulin gene expression. Trends Endocrinol Metab 8:145–151
16. **Edlund H** 1998 Transcribing pancreas. Diabetes 47:1817–1823
17. **Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD** 2001 Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3:778–784
18. **Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay RD** 2001 Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292:1389–1394
19. **Heller RS, Kieffer TJ, Habener JF** 1997 Insulinotropic glucagon-like peptide I receptor expression in glucagon-producing α-cells of the rat endocrine pancreas. Diabetes 46:785–791
20. **Daniel PB, Habener JF** 1998 Cyclical alternative exon splicing of transcription factor cyclic adenosine monophosphate response element-binding protein (CREB) messenger ribonucleic acid during rat spermatogenesis. Endocrinology 139:3721–3729
21. **Lu M, Seufert J, Habener JF** 1997 Pancreatic β-cell-specific repression of insulin gene transcription by CCAAT/enhancer-binding protein β. Inhibitory interactions with basic helix-loop-helix transcription factor E47. J Biol Chem 272:28349–28359
22. **Stoffers DA, Stanojevic V, Habener JF** 1998 Insulin promoter factor-1 gene

mutation linked to early-onset type 2 diabetes mellitus directs expression of a dominant negative isoprotein. J Clin Invest 102:232–241
23. **Miller CP, McGehee REJ, Habener JF** 1994 IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. EMBO J 13:1145–1156
24. **Schreiber E, Matthias P, Muller MM, Schaffner W** 1989 Rapid detection of octamer binding proteins with 'mini-extracts,' prepared from a small number of cells. Nucleic Acids Res 17:6419
25. **Holz GG, Kuhtreiber WM, Habener JF** 1993 Pancreatic β-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). Nature 361:362–365
26. **Weir GC, Mojsov S, Hendrick GK, Habener JF** 1989 Glucagon like peptide I (7–37) actions on endocrine pancreas. Diabetes 38:338–342
27. **Jonsson J, Carlsson L, Edlund T, Edlund H** 1994 Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371:606–609
28. **Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Krasik A** 2000 Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 6:568–572
29. **Josephson R, Muller T, Pickel J, Okabe S, Reynolds K, Turner PA, Zimmer A, McKay RD** 1998 POU transcription factors control expression of CNS stem cell-specific genes. Development 125:3087–3100
30. **Hussain MA, Lee J, Miller CP, Habener JF** 1997 POU domain transcription factor brain 4 confers pancreatic α-cell-specific expression of the proglucagon gene through interaction with a novel proximal promoter G1 element. Mol Cell Biol 17:7186–7194
31. **Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF** 1987 Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. Proc Natl Acad Sci USA 84:3434–3438
32. **Skoglund G, Hussain MA, Holz GG** 2000 Glucagon-like peptide 1 stimulates insulin gene promoter activity by protein kinase A-independent activation of the rat insulin I gene cAMP response element. Diabetes 49:1156–1164
33. **Buteau J, Roduit R, Susini S, Prentki M** 1999 Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in β (INS-1)-cells. Diabetologia 42:856–864
34. **Montrose-Rafizadeh C, Avdonin P, Garant MJ, Rodgers BD, Kole S, Yang H, Levine MA, Schwindinger W, Bernier M** 1999 Pancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells. Endocrinology 140:1132–1140
35. **Garcia-Flores M, Zueco JA, Alvarez E, Blazquez E** 2001 Expression of glucagon-like peptide-1 (GLP-1) receptor and the effect of GLP-1-(7–36) amide on insulin release by pancreatic islets during rat ontogenic development. Eur J Biochem 268:514–520
36. **Gribble FM, Ashcroft FM** 2000 Sulfonylurea sensitivity of adenosine triphosphate-sensitive potassium channels from β cells and extrapancreatic tissues. Metabolism 49:3–6
37. **Ashcroft FM, Gribble FM** 2000 Tissue-specific effects of sulfonylureas: lessons from studies of cloned K(ATP) channels. J Diabetes Complications 14:192–196
38. **Seufert J, Weir GC, Habener JF** 1998 Differential expression of the insulin gene transcriptional repressor CCAAT/enhancer-binding protein β and transactivator islet duodenum homeobox-1 in rat pancreatic β cells during the development of diabetes mellitus. J Clin Invest 101:2528–2539
39. **Donath MY, Gross DJ, Cerasi E, Kaiser N** 1999 Hyperglycemia-induced β-cell apoptosis in pancreatic islets of *Psammomys obesus* during development of diabetes. Diabetes 48:738–744
40. **Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, Usellini L, Nano R, Bonini P, Bertuzzi F, Marlier LN, Davalli AM, Carandente O, Pontiroli AE, Melino G, Marchetti P, Lauro R, Sesti G, Folli F** 2001 High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. Diabetes 50:1290–1301
41. **Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY** 2001 Glucose induces β-cell apoptosis via upregulation of the Fas receptor in human islets. Diabetes 50:1683–1690
42. **Slack JM** 1995 Developmental biology of the pancreas. Development 121: 1569–1580
43. **Al-Abdullah IH, Ayala T, Panigrahi D, Kumar RM, Kumar MS** 2000 Neogenesis of pancreatic endocrine cells in copper-deprived rat models. Pancreas 21:63–68
44. **Jones LC, Clark A** 2001 β-Cell neogenesis in type 2 diabetes. Diabetes 50(Suppl 1):S186–S187
45. **Bernard C, Berthault MF, Saulnier C, Ktorza A** 1999 Neogenesis vs. apoptosis as main components of pancreatic β cell mass changes in glucose-infused normal and mildly diabetic adult rats. FASEB J 13:1195–1205
46. **Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I** 1996 Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J Clin Invest 97:1647–1654
47. **Wang H, Maechler P, Ritz-Laser B, Hagenfeldt KA, Ishihara H, Philippe J, Wollheim CB** 2001 Pdx1 level defines pancreatic gene expression pattern and cell lineage differentiation. J Biol Chem 276:25279–25286
48. **Hussain MA, Miller CP, Habener JF** 2001 Brn-4 transcription factor expression targeted to the early developing mouse pancreas induces ectopic glucagon gene expression in insulin-producing β-cells. J Biol Chem 277:16028–16032
49. **Teitelman G, Alpert S, Polak JM, Martinez A, Hanahan D** 1993 Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development 118:1031–1039
50. **Polak M, Bouchareb-Banaei L, Scharfmann R, Czernichow P** 2000 Early pattern of differentiation in the human pancreas. Diabetes 49:225–232